



1973

## The effects of aging and transformation on the DNA, RNA, protein, and hydroxyproline content of fibroblasts (WI 38) in culture

James Michael Eichner  
*University of the Pacific*

Follow this and additional works at: [https://scholarlycommons.pacific.edu/uop\\_etds](https://scholarlycommons.pacific.edu/uop_etds)

 Part of the [Life Sciences Commons](#)

---

### Recommended Citation

Eichner, James Michael. (1973). *The effects of aging and transformation on the DNA, RNA, protein, and hydroxyproline content of fibroblasts (WI 38) in culture*. University of the Pacific, Thesis.  
[https://scholarlycommons.pacific.edu/uop\\_etds/1812](https://scholarlycommons.pacific.edu/uop_etds/1812)

This Thesis is brought to you for free and open access by the Graduate School at Scholarly Commons. It has been accepted for inclusion in University of the Pacific Theses and Dissertations by an authorized administrator of Scholarly Commons. For more information, please contact [mgibney@pacific.edu](mailto:mgibney@pacific.edu).

THE EFFECTS OF AGING AND TRANSFORMATION ON THE DNA, RNA,  
PROTEIN, AND HYDROXYPROLINE CONTENT OF  
FIBROBLASTS (WI 38) IN CULTURE

---

A Thesis  
Presented to  
the Faculty of the Graduate School  
University of the Pacific

---

In Partial Fulfillment  
of the Requirements for the Degree  
Master of Science

---

by  
James Michael Eichner  
July, 1973



LIBRARY

JAN 14 1974

UNIVERSITY OF THE PACIFIC

274638

Science

This thesis, written and submitted by

James Michael Eichner

is approved for recommendation to the Committee  
on Graduate Studies, University of the Pacific.

Department Chairman or Dean:

Carl C. Ruedel

Thesis Committee:

Donald M. Pace Chairman

Donald G. Shirachi

Frank Palmer

Dated July 24, 1973



#### ACKNOWLEDGEMENT

The author wishes to express appreciation to Dr. Donald M. Pace for guidance in this study; to Dr. Donald Y. Shirachi for his help in this study; and to Mrs. Jeanne Eichner for her special technical assistance.

## TABLE OF CONTENTS

INTRODUCTION . . . . .	1
Nature of the Aging Process. . . . .	1
The Study of Aging <u>In Vitro</u> . . . . .	2
The Model System for the Study of Aging. . . . .	3
Previous Investigation of Human Fibroblasts in Culture . . . . .	11
Purpose of the Present Investigation . . . . .	14
MATERIALS AND METHODS. . . . .	16
Origin of the Cell Strain Used . . . . .	17
Cultivation of the Cells . . . . .	17
Sampling and Counting of Cells . . . . .	18
Extraction Procedure . . . . .	20
Determination of RNA . . . . .	23
Determination of DNA . . . . .	24
Determination of Protein . . . . .	25
Determination of Hydroxyproline. . . . .	25
Methods of Analysis of Data. . . . .	28
RESULTS. . . . .	30
Growth of the Cell Strain. . . . .	31
Cellular DNA Content . . . . .	39
Cellular RNA Content . . . . .	45
Cellular Protein Content . . . . .	45
Cellular Hydroxyproline Content. . . . .	45
Hydroxyproline Content of the Medium . . . . .	51

DISCUSSION . . . . .	54
SUMMARY. . . . .	61
BIBLIOGRAPHY . . . . .	63

## LIST OF FIGURES

FIGURE	PAGE
1. Diagrammatic representation of the theory of <u>in vitro</u> cell proliferation. . . . .	6
2. Flow sheet procedure for the separation of RNA, DNA, and protein. . . . .	22
3A. WI 38 fibroblasts in the log phase of growth. B. WI 38 fibroblasts at confluency. . . . .	33
4A. WI 38 fibroblasts showing internal structure. B. WI 38 fibroblasts in Phase III . . . . .	36
5. SV <sub>40</sub> transformed WI 38 cells . . . . .	38
6. The number of WI 38 fibroblasts in confluent cultures at various generations. . . . .	41
7. The effect of aging and transformation on the DNA content of fibroblasts . . . . .	44
8. The effect of aging and transformation on the RNA content of fibroblasts . . . . .	47
9. The effect of aging and transformation on the protein content of fibroblasts . . . . .	50
10. The effect of aging and transformation on the hydroxyproline content of fibroblasts. . . . .	53

## LIST OF TABLES

TABLE	PAGE
I. The Effects of Aging and Transformation on the DNA and RNA Content of Fibroblasts . . . . .	42
II. The Effects of Aging and Transformation on the Hydroxyproline and Protein Content of Fibroblasts.	48

## INTRODUCTION



## INTRODUCTION

### Nature of the Aging Process

The study of the aging process is the investigation as to how the passage of time affects cells, organs, and organisms. Aging is a very complex and incompletely understood phenomenon. This is reflected by the number of theories attributing aging to a variety of causative factors such as: (1) the somatic mutations occurring spontaneously or produced by ionizing radiation, which are thought to have some effect on aging but are not responsible for the normal process (Smith, 1962); (2) the alteration of macromolecules as the cells of an organism age forming neoantigens and functioning in the autoimmune reaction (Walford, 1967); the Cross-linkage theory which maintains that large molecules necessary for life processes, such as deoxyribonucleic acid (DNA) and collagen are progressively immobilized in all cells and tissues by cross-linkage (Bakerman, 1969). Aging has also been studied in relation to the self-destructive "programmed death" characteristic of some parts of embryological development (Bakerman, 1969). Moreover, senescent changes involve different kinds of cells and tissues in the organism and therefore various mechanisms must occur. For example, the aging of postmitotic cells, such as neurons and cardiac cells probably proceeds by a different mechanism than the proliferating tissues, such as the skin, the gut lining, and the blood forming elements. It is apparent that there is probably no single aging process, but a series of aging

processes which natural selection would tend to synchronize even if the causes were physiologically independent (Krohn, 1966).

#### The Study of Aging In Vitro

The effects of the aging process had been observed in cells cultivated in vitro as early as 1910 when Alexis Carrel observed that the time elapsing prior to cell migration from explanted tissue fragments was longer with those of adult origin than those of embryonic origin. Carrel in 1923 reported that the serum of older animals had an inhibiting action on the growth of fibroblasts in culture. The serum of younger animals had an inhibitory action also, but to a much lesser extent. In the development of tissue culture from the beginning of the century until the 1930's, it was generally regarded that cells in culture, irrespective of type of tissue, usually had a finite lifespan of less than a year. It was concluded by Hayflick (1965) that the primitive techniques used by the early tissue culturists were the cause of the short term cultivation of cells in vitro. One possible exception to this generalization was the work of Carrel who kept a population of cells derived from embryonic chick heart in serial cultivation for 34 years (Parker, 1961). Serious doubt has been cast upon this experiment for two reasons: (1) this has not been repeated in any laboratory with chick heart fibroblasts even with more modern and sophisticated cell culture techniques; and (2) the method



of preparation of the chick embryo extract, used as a source of nutrient for the culture, permitted cell survival from the embryo which contributed new, viable, embryonic cells to the chick heart strain at each subcultivation or feeding (Hayflick, 1961).

Gey (1936) and Earle (1943) reported great advances in the methodology of tissue culture. They demonstrated that cell populations derived from a number of mammalian tissues could be maintained in a proliferative state in tissue culture for indefinite periods of time. Since their work, cell populations with the capacity to multiply in vitro for indefinite periods of time have been spontaneously derived from over 200 mammalian tissues.

Hayflick and Moorhead (1961) reported only limited success in developing strains of human cells in tissue culture that could be cultivated for long periods and still have the normal diploid chromosomal configuration. When they isolated cultures of human fibroblasts, they found that in all cases where the cells remained in the normal diploid chromosomal configuration, the cultures underwent 40-50 generations in ten months before degeneration and final termination. This limitation of cell division has been ascribed (Hayflick and Moorhead, 1961) as a model system for the study of aging.

#### The Model System for the Study of Aging

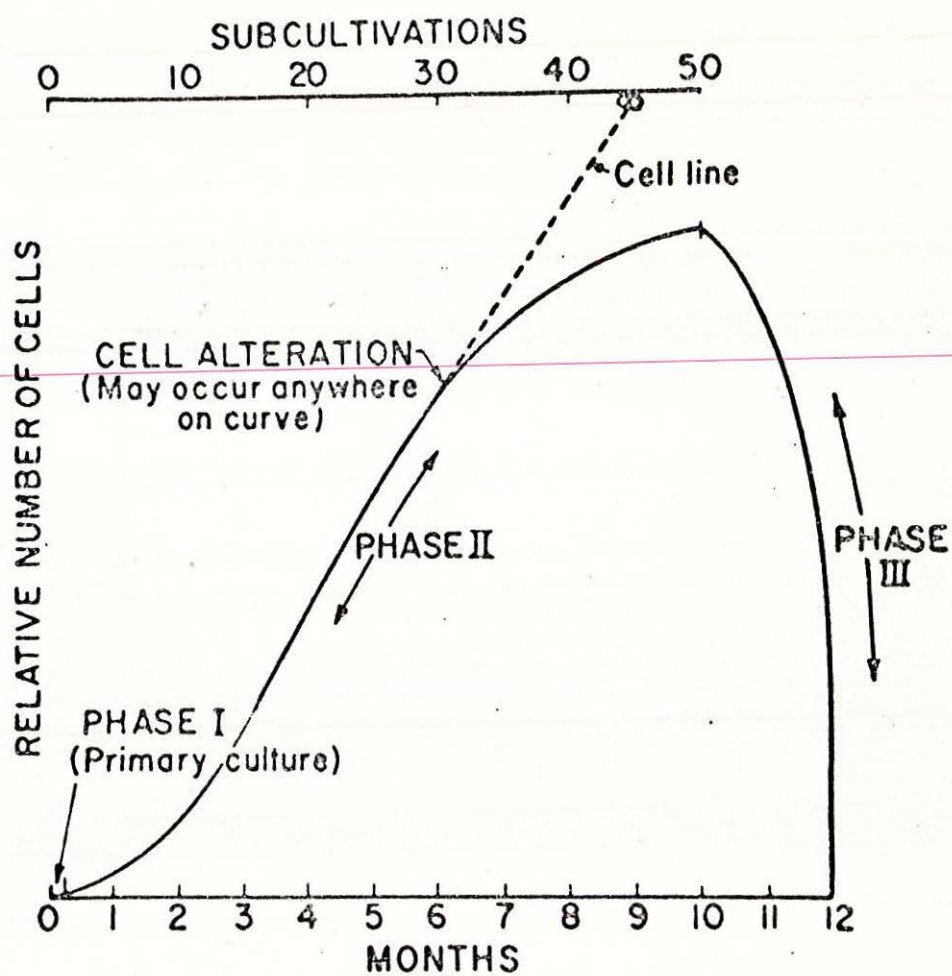
The report of Hayflick and Moorhead in 1961 focused

attention on the fact that two basic types of cell cultures exist, those of a diploid chromosomal configuration with a finite lifespan, and those of a heteroploid chromosomal configuration with an apparent infinite lifespan. Figure 1 shows, graphically, the growth of diploid cells in culture. When a culture is first initiated from the progenator tissue, it is called a primary culture. In one to three weeks the cells grow and divide forming a continuous or confluent monolayer on the glass substrate. This period of time is considered Phase I of cellular proliferation. The culture is then subcultivated, that is, the cells are suspended by trypsinization and removed from the culture vessel and half are placed in another vessel. Each cell then divides once and the two new cultures contain a confluent sheet of cells. In this sense, each subcultivation is referred to as one generation. This period of rapid proliferation in which many subcultivations take place, is called Phase II. After several generations, the cellular growth slows, i.e., a gradual decline in mitotic activity with increased generation time, much cellular debris occurs, and there is a gradual cessation of cellular activity. This period is Phase III and commences when the cells are incapable of proliferation on subcultivation and the culture degenerates completely.

During Phase II of some cell cultures, notably those of mouse origin, a cell alteration occurs called a transformation. This can occur at any time during Phase II. The cells

Figure 1. Diagrammatic representation of the theory of in vitro cell proliferation (Hayflick and Moorhead, 1961).

Figure 1





that result from the transformation have a different morphology than their progenator cells. They are heteroploid and can be subcultivated indefinitely. When inoculated into a suitable host, these cells usually cause the formation of tumor masses. Thus, transformed cells are usually considered malignant.

A special terminology has been established for normal and transformed types of cultures (Hayflick and Moorhead, 1961). Cell populations first released from tissue and cultivated in vitro are called primary cultures. When these undergo further proliferation, sufficient to be subcultured, this is referred to as a serially propagated cell culture and is designated as a cell strain. The cell cultures which undergo a transformation during Phase II and are permanently proliferating are designated as a cell line.

It has been demonstrated that the Phase III phenomenon is not the result of the cellular environment, that is, external influences on the cells. Hayflick and Moorhead (1961) performed various experiments with degenerating cells changing medium components, further subcultivations, and crowding into one culture a number of degenerating cultures. All attempts failed at extending the life of the cells. It is apparent that the medium composition plays no role in causing degeneration. Portions of the same pool of medium support luxuriant growth of cells of earlier generations while the older cultures degenerate.

The detrimental effect of possible contaminating microorganisms was also discounted by Hayflick and Moorhead (1961). The cultures were periodically monitored for the presence of pleuropneumonia-like organisms (PPL0). This was considered important because this is perhaps the only microorganism that could pass through the filters used in the sterilization of the medium. No PPL0 was ever detected in the cultures. Also, attempts were made to detect the presence of latent viruses in the cells. Spent medium both with and without cells was added to many types of tissue cultures and injected into laboratory animals with consistently negative results. Medium and cells from cultures in Phase III were particularly tested for the possible unmasking of latent viruses. In no case was cytopathology observed in any cultures either before or during Phase III. Cells of different ages were also stained by various methods for possible virus inclusions but none were observed.

It has been presumed that the number of subcultivations is equivalent to the number of cell doublings. In order to prove this, it was necessary to accurately determine the total number of cell doublings that occur until the end of Phase III. This is accomplished best by keeping the cell population in continuous logarithmic growth in a suspension culture. However, no one has been able to successfully cultivate any human diploid cell strain in suspension culture. An alternative method was used to determine the



correlation between the termination of Phase III and the total number of accumulated cell doublings. Sister cultures were taken at the 14th passage and subcultivated at two different split ratios, 2 : 1 and 10 : 1. In this way, the cells of the 10 : 1 split ratio were kept in the log phase for a longer time than the 2 : 1 split ratio. It was determined that the total calendar time until the commencement of Phase III was similar for both cultures. However, the cells kept in almost constant log phase accumulated somewhat more generations than the 2 : 1 split ratio before the termination of Phase III. The split ratio therefore appears to effect the total number of generations. Nevertheless, it is evident that with a 2 : 1 split ratio in 50 subcultivations, approximately 50 cell doublings occurs.

It was questioned as to whether each cell in the population is endowed with the "50 passage potential," or does each cell of the population have a different random passage potential which results in an average of 50 passages for the entire population. To determine this, cloning experiments were performed. Three clones were selected at random, allowed to propagate until confluency, and subsequently subcultured until the termination of Phase III. It was observed that the total number of cell doublings was approximately the same for the three clones. It is evident that each cell of the population has the same doubling potential.

In order to determine if the Phase III phenomenon

was intracellularly determined, as experiment was devised using mixed populations of cells at different ages. A male human diploid cell strain of the 49th passage (Phase III) containing many metabolically active, non-dividing cells, was mixed with a suspension of actively dividing female human diploid cells of the 13th passage (Phase II). Seventeen passages after mixing, cells in metaphase were examined and no male cells were found. The female cells entered Phase III at approximately the same passage as an unmixed control culture. Thus, the "old" cells had no detectable effect on the "young" cells or vice versa.

It has been established that cell strains developed from human embryos have a division potential of  $50 \pm 10$  generations (Hayflick and Moorhead, 1961). It was of interest to compare the occurrence of Phase III in cell strains of embryonic origin and those of adult origin. A number of strains were developed from adult donors of various ages. In all cases, Phase III occurred at approximately 20 generations. Since the donors ranged in age from 26 to 87 years, no correlation could be made between age and the number of passages accruing until Phase III. However, it is clear that there is significant difference between cultures of adult and fetal origin.

From the foregoing, it is evident that the Phase III phenomenon is not the result of deficiencies in medium composition, microorganisms, conditions of cell culture, or



the elaboration of toxic products by the cells. This degeneration of embryonic cells in culture has been employed as a model system for the study of aging.

#### Previous Investigation on Human Fibroblasts in Culture

Since Hayflick and Moorhead first established cultivated fibroblasts as a model system for the study of aging, many morphological and biochemical investigations have taken place. Most of the research has been with the strain of fibroblasts originally isolated by Hayflick (1965), the so-called WI 38. The "WI" refers to the Wistar Institute where the strain was isolated, and the "38" stands for the thirtyeighth strain of cells isolated. Other strains of fibroblasts have been established not only from human embryonic lung, but from gingiva, skin, and kidney (Macek, 1967) and from chick embryos (Ryan and Quinn, 1971). These strains meet the criteria for aging cells in culture, i.e., they exhibit Phases I, II, and III, and they have been used to investigate the aging process.

In morphological studies by Robbins et al. (1970), it has been reported that major changes in lysosomes occurs as the fibroblasts are propagated in culture. The lysosomes increase progressively in both size and number, and show profound "degenerative" changes. The lysosomes gradually transform to residual bodies which increase in size and number. Also, there is an accompanying decrease in free cytoplasmic polysomes and an accumulation of glycogen.

Brandes et al. (1972) have shown that there was an initial increase in lysosomal content when the fibroblasts were first established in vitro, as well as the appearance of a large number of filaments and microtubules. Further studies of the propagation of the fibroblasts in vitro showed an increase in lysosomal content as the cells age.

The morphological investigation of lysosomes correlates well with biochemical findings. Cristofalo (1970) fractionated the WI 38 cells at various passage levels and compared the subcellular distribution of acid phosphatase and  $\beta$ -glucuronidase as a function of age. All fractions showed an increase in activity with aging, with the greatest portion of activity in the fraction containing the lysosomes. There was also a pronounced increase in activity in both acid phosphatase and  $\beta$ -glucuronidase in the supernatant fraction. This suggested the possibility that the lysosomal enzymes were less tightly bound in older cells and this may account for the increased activity.

Macieira-Coelho et al. (1966) using growth curves and autoradiography have shown an increased heterogeneity in the length of the division cycle in late passage cells. This is due principally to an increase in the extent of the  $G_1$  and  $G_2$  periods of the cell cycle and the fact that the fraction of cells included in DNA synthesis decreased as a function of culture age. This suggests that the limitation on lifespan is not derived from a uniform, gradual decrease in the



capacity for reproduction in Phase III, but due to cells spanning the spectrum between the two extremes of complete inhibition and normal division cycle.

Glucose metabolism has been studied extensively in human diploid cells. The cells in culture utilize glucose as their principle source of carbon producing primarily lactate which is excreted into the medium. Cristofalo (1970) has concluded that the cellular aging process in WI 38 cells is not accompanied by any significant changes in their respiratory or glycolytic capacities. He has suggested that cellular senescence does not result from the inability to utilize available energy sources.

The glycogen content of the WI 38 cells shows a pronounced variation at different generations (Cristofalo, 1970). A large increase of cellular glycogen as the cultures age was noted and confirmed by ultrastructural studies of Robbins et al. (1970).

Various aspects of lipid metabolism have been studied during aging. Kritchevsky et al. (1970) have demonstrated a significant increase in lipid content in late passage cells. However, no age-related differences in cholesterol content or its rate of biosynthesis were noted. Increased cholesterol biosynthesis was observed in transformed cells as compared with diploid fibroblasts.

Investigations by Hay and Strehler (1967) indicates that there appear to be no changes in cellular permeability

to amino acids as the cultures age. Cristofalo et al. (1970) have compared protein content of human diploid cells during aging and found that the total protein content per cell was similar for all passages. Ryan and Quinn (1971) have compared the nucleohistone content of embryonic chick fibroblasts in culture and found no changes in the histone : DNA ratios as a function of age.

Cristofalo et al. (1970) have determined DNA and RNA content during the aging of the WI 38 fibroblasts. Their data show that the DNA content per cell did not vary with age of the culture. However, the RNA content increased approximately three-fold in senescent cultures as compared to young cells.

Macek et al. (1967) working with fibroblasts derived from human embryonic skin, gingiva, and umbilical tissue, have measured hydroxyproline content as an indication of collagen content. They found a constant hydroxyproline content until generation 40 and then a gradual decline. Houch et al. (1971) reported a great loss of the ability of fibroblasts to produce collagen after generation 35.

#### Purpose of the Present Investigation

This study was designed to investigate certain aspects of the aging process using the WI 38 fibroblasts as a model system, in relation to WI 38 cells which have been transformed by Simian Virus 40 (Girardi et al., 1965). Cristofalo et al. (1970) have established that as the DNA and protein



content of the cells remained constant, the RNA increased as a function of age. Since the WI 38 cells are fibroblasts, and their chief function is to produce connective tissue, the content of the connective tissue collagen was studied in relation to DNA, RNA, and protein content as the cells undergo the aging process. Cristofalo et al. (1970) has speculated that RNA triples in aged cells as the DNA and protein content remained constant because there is an error in transcription of the RNA. This defective RNA produces enzymes incapable of normal function. The consequent accumulation of substrate molecules then results in derepression of RNA synthesis, and more RNA is formed. It might be expected that the collagen content would increase with age because RNA increases. However, the defective RNA might produce defective enzymes which will not produce collagen, and the collagen content would decrease with age.

With the transformed WI 38 cells, it was predicted that the DNA content would be higher than in the normal cells due to an aneuploid chromosomal configuration; the RNA content would be greater due to more protein synthesis for cell division; and the total protein content would be less because the transformed cells are smaller than the normal cells. Also, it was predicted that the collagen content would be decreased in transformed cells because of their loss of differentiation during the transformation process (Harris, 1964).

## MATERIALS AND METHODS

## MATERIALS AND METHODS

### Origin of the Cell Strain Used

In this study human diploid fibroblasts were employed. They were derived from a human embryonic female lung obtained from a surgical abortion after approximately 3 months gestation (Hayflick, 1965). The culture was prepared by freeing the cells by mincing or fragmenting the tissue. The lung tissue was placed in a Petri dish containing nutrient medium and cut with scissors or paired scalpels until the size of each piece was approximately 1-4 mm<sup>3</sup>. The contents of the dish were emptied into a pyrex Blake bottle and incubated at 37°C. After growth was first observed, the spent medium and any tissue fragments present were discarded and new medium placed on the cells. As soon as a confluent sheet of cells formed, the culture was subcultivated. After several subcultivations, the cells were preserved with liquid nitrogen. Prior to using the cells they were thawed, placed in a culture flask with nutrient medium, and allowed to propagate.

The WI 38 fibroblasts and SV<sub>40</sub> transformed WI 38 cells used in this study were obtained from the laboratory of Dr. L. Hayflick, Stanford University, Palo Alto, California, as confluent cultures.

### Cultivation of the Cells

The cells were cultivated in pyrex T-60 flasks (Earle, 1951) which have a surface area of approximately 60 cm<sup>2</sup>. They were maintained on a medium consisting of Basal Medium Eagle (Diploid) and 10% Fetal Calf Serum (Grand Island



Biological Company, Berkeley, California). The cultures were allowed to grow to confluency, i.e., the flask was completely covered with cells and very few cells were in mitosis at this time. Confluency occurred in approximately 4 days in younger cultures but took up to 10 days in older cultures. At confluency, the cultures were subcultured 2 : 1, that is, half the cells were removed and placed in another flask. This was done by removing the old medium from the culture and placing 5 ml of 0.25% trypsin (Difco Laboratories, Detroit, Michigan) solution in calcium and magnesium free phosphate buffered saline (CMF PBS; Merchant, Kahn, and Murphy, 1964). Following approximately 1 minute exposure, the trypsin solution was decanted and the culture was incubated at 37°C. After 15 minutes, the cells were observed sliding down the glass surface. A few ml of growth medium was added, and the culture gently shaken to suspend the cells. A graduated pipette with a rubber bulb was used to aspirate and expel the cell suspension several times to break up any clumps. Half of the suspension was placed in another flask and enough growth medium added to make up 12 ml in each flask. An atmosphere of 95% air and 5% carbon dioxide was added, and the cultures were stoppered and incubated at 37°C.

#### Sampling and Counting of Cells

Samples of cells were taken at different generations during the aging process. The samples were taken when the cultures were confluent and few cells were in mitosis. Each sample consisted of the contents of one T-60 flask. The



culture medium was decanted into a test tube and the cell layer was washed with approximately 15 ml of warm balanced salt solution. One and one-half ml of 0.25% trypsin in CMF PBS was added, the culture swirled to distribute the trypsin solution, and incubated at 37°C. After 15 minutes, the culture was given a brisk shake and the cells were observed sliding down the glass surface. The cells were suspended in approximately 3 ml of CMF PBS and removed by means of a pipette and put into a 12 ml graduated glass centrifuge tube. The flask was rinsed twice with CMF PBS and the rinsings placed in the centrifuge tube. The volume of the cell suspension was made up to 10 ml with CMF PBS. With a 1 ml pipette, the cells were evenly suspended and 1 ml was removed and placed in a 25 ml graduated cylinder for counting. The volume was made up to 15 ml with counting fluid. This is an isotonic calcium and magnesium free solution containing Versene® and Darvan® (R. T. Vanderbilt, Inc., New York, New York), a dispersing agent. The cell suspension in counting fluid was incubated at 37°C for 10 minutes and counted by means of the Coulter electronic counter, Model B (Coulter Electronics, Miami, Florida). The number of cells in the remaining 9 ml suspension was calculated. The suspension was centrifuged at 1000 RPM for 15 minutes on an International Clinical Centrifuge (International Equipment Co., Needham Hts., Massachusetts). The supernatant was decanted into another test tube and the samples were frozen

at  $-10^{\circ}\text{C}$  for further determinations.

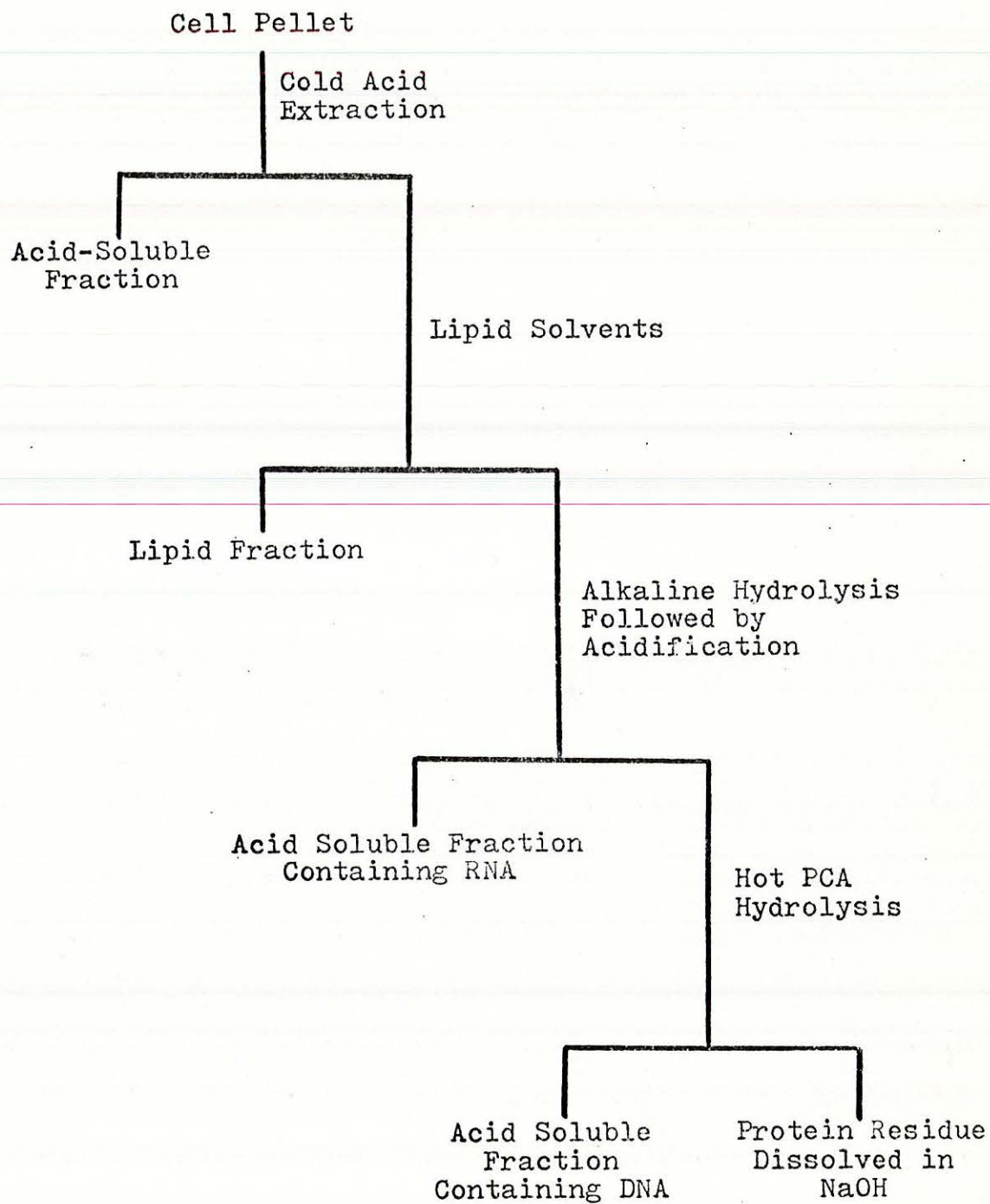
#### Extraction Procedure

RNA, DNA, and protein were extracted from the cells by a modified Schmitt-Thannhauser procedure (1945) as outlined by Merchant *et al.* (1964). This is represented by the flow sheet procedure in Figure 2. The cell pellet was extracted in 1 ml of cold ( $4^{\circ}\text{C}$ ) 0.5N perchloric acid (PCA) for 30 minutes, and centrifuged. This, and all centrifugations, were done in a Sorvall Superspeed refrigerated centrifuge Model RC-2 (Ivan Sorvall Inc., Norwalk, Connecticut) at 5000 RPM at  $4^{\circ}\text{C}$ . This extraction removes sugars, other polysaccharides and nucleotide coenzymes that would effect the assays for DNA and RNA. The residue was washed with 0.2N potassium acetate in ethanol, and extracted twice with 3 : 1 ethanol/ether mixture at  $50^{\circ}\text{C}$  for 30 minutes. After the lipid extraction, the residue was dissolved by suspending it in potassium hydroxide (0.1N) for 16-18 hours at  $37^{\circ}\text{C}$ . This alkaline hydrolysis was used to separate RNA from DNA and protein. It is thought (Hutchison and Monro, 1961) that the RNA undergoes hydrolysis in preference to DNA. This solubilized cell material was then acidified with 1 ml of a mixture of 0.5N PCA and 6N hydrochloric acid (100:3.4), and the DNA and protein allowed to precipitate at  $4^{\circ}\text{C}$  for 30 minutes. The RNA dissolves in the cold acid whereas DNA and protein do not. The residue was suspended in 1 ml of 0.5N PCA and heated to  $90^{\circ}\text{C}$  for 15 minutes in a water bath. The DNA

Figure 2. Flow sheet procedure for separation of RNA, DNA, and protein.



Figure 2



was hydrolyzed, making it soluble in the acid. The remaining residue is protein which was dissolved in 2.5 ml of 1N sodium hydroxide. Recovery experiments were performed for the extraction procedure. Known quantities of herring sperm DNA, yeast RNA, bovine serum albumin, and calf skin collagen (Sigma Chemical Co., St. Louis, Missouri), were subjected to the extraction procedure and subsequently the quantities of each determined by its respective assay. These results were compared with assays of known quantities of each without extraction and the percent recoveries were determined. The recoveries were: DNA, 99-100%; RNA, 98-100%; protein, 66%; and collagen, 91%. All data were corrected for recovery.

#### Determination of RNA

The quantitation of RNA was determined by a modification of the method of Mejbaum (1939) as presented by Merchant et al. (1964). This involves the measurement of the pentose content by the orcinol (5-methyl resorcinol, Matheson, Coleman and Bell, Norwood, Ohio) reaction. A stock orcinol reagent solution was prepared by dissolving 2.7 g of ferric ammonium sulfate and 4 g of orcinol (recrystallized from benzene) in 100 ml of double distilled water. This was stored in the cold at 4°C. The orcinol reagent was prepared by adding 5 ml of orcinol stock solution to 85 ml of concentrated hydrochloric acid and bringing it to 100 ml with double distilled water. Three ml of this solution were added to 1 ml of

sample and heated in a boiling water bath for 20 minutes. The samples were cooled and read in a Beckman Spectrophotometer Acta CII (Beckman Instruments , Inc., Fullerton, California) at 670 mu. A standard curve was constructed using quantities of yeast RNA, ranging from 0 to 100 ug/ml of aqueous solution. The RNA concentration in the sample was computed by means of the standard curve.

#### Determination of DNA

The quantitative determination of DNA was accomplished by the measurement of deoxyribose by the diphenylamine (Matheson, Coleman and Bell, Norwood, Ohio) reaction originally described by Dische and modified by Burton (1956). A diphenylamine stock solution was prepared by adding 1.5 g of diphenylamine (recrystallized twice from ethanol) and 1.5 ml of sulfuric acid to 100 ml of glacial acetic acid (redistilled). Just before use, 0.1 ml of acetaldehyde (redistilled; 16mg/ml aqueous solution) was added to 20 ml of the diphenylamine stock solution to prepare the diphenylamine reagent solution. Three ml of this solution was added to 1 ml of sample which was stoppered and incubated at 37°C for 18 hours. The samples were then read on a Beckman Spectrophotometer at 600 mu. A standard curve was constructed using quantities of herring sperm DNA ranging from 0 to 50 ug/ml dissolved in 5 mM sodium hydroxide. The values of the unknown samples were determined by means of the standard curve.

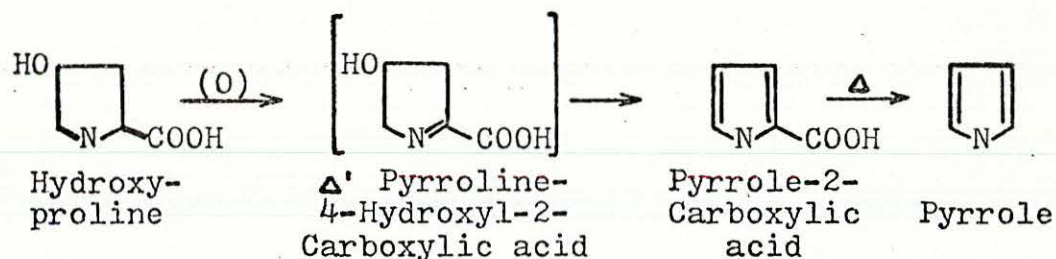


### Determination of Protein

The quantitative determination of protein was according to the method of Lowry (1951), a modification of the Folin-Ciocalteu method. This is based on the color reaction of the aromatic amino acids tyrosine and tryptophan with the Folin-Ciocalteu Phenol reagent. One-half ml of 0.5% copper sulfate in aqueous solution and 0.5 ml of 1% sodium potassium tartrate in aqueous solution were brought to 50 ml with 2% sodium carbonate in 0.1N sodium hydroxide, and 1 ml of this was added to 1.5 ml of sample. After 10 minutes, 0.1 ml of the Folin-Ciocalteu Phenol reagent (Van Waters and Rogers, San Francisco, California), diluted 1 : 1 in water was added to each sample with stirring. The samples were read after 30 minutes on a Beckman Spectrophotometer at 750 m $\mu$ . A standard curve was constructed using bovine serum albumin ranging from 0 to 60  $\mu$ g per sample. The values of the unknown samples were determined by means of the standard curve.

### Determination of Hydroxyproline

Since nearly all the hydroxyproline (HPr) of the cell is found in collagen, the results of the HPr determination are indicative of the collagen content of the cell. The assay for HPr used in this investigation is that of Prockop and Udenfriend (1960) based on the oxidation of the imino acid to a carboxylated intermediate, which is decarboxylated to pyrrole forming a chromophore with p-dimethylaminobenzaldehyde.



Both the cells and an aliquot of the medium were assayed for HPr. The cellular HPr was found both in the protein solution derived from the cells after the extraction procedure and in the supernatant which resulted after the cells were removed from the flask in suspension and centrifuged.

An equal volume of concentrated hydrochloric acid was added to the samples. They were sealed in screw cap test tubes and hydrolyzed in an oven at 125°C for 24 hours. This hydrolysis frees the hydroxyproline from the collagen. The hydrolysates of the protein solution and supernatant were placed together since they represented the total cellular HPr. This was concentrated to 5 ml by evaporation. A 2 ml aliquot of the medium hydrolysate was brought to 5 ml with water. Pigments and particulate matter were removed from the samples by mixing with Humin Precipitant which is a mixture of Dowex-1 (a strongly basic anion exchange resin; chloride form, 200-400 mesh) and Norite A (Pfanstiehl Laboratories, Inc., Waukegan, Illinois), and centrifuging at 2400 RPM for 15 minutes. Four ml of the clear supernatant



were removed and placed in a large test tube. One drop of 1% phenolphthalein was added and the sample was titrated to the phenolphthalein endpoint with 10N and 0.1N potassium hydroxide. The samples were saturated with potassium chloride, and 1 ml of a 10% DL-alanine (Matheson, Coleman and Bell, Norwood, Ohio) solution of pH 8.7 and 2 ml of a potassium borate buffer (61.84 g boric acid and 225 g of potassium chloride in 1 liter of distilled water, pH 8.7) were added.

The samples were then oxidized with 2 ml of 0.2M Chloramine T (Matheson, Coleman and Bell, Norwood, Ohio) solution in Methyl Cellosolve for 20 minutes. The oxidation was stopped by adding 6 ml of 3.6 M sodium thiosulfate. Ten ml of toluene were added to each sample and shaken for 1 minute to remove any possible interfering materials. The toluene was removed and discarded. The samples were placed in a boiling water bath for 30 minutes. They were cooled, 5 ml of toluene added, and shaken for 1 minute. Two and one-half ml of the toluene phase was removed and 1.0 ml of Ehrlich's Reagent (24 g of dimethylaminobenzaldehyde and 5.48 ml of sulfuric acid in 80 ml of absolute ethanol) was added. After 15 minutes the samples were read on a Beckman Spectrophotometer at 560 mμ. A standard curve was constructed using hydroxyproline (Matheson, Coleman and Bell, Norwood, Ohio) ranging from 0 to 0.125 umoles per sample. The values of the samples were determined by means of the standard curve.

The specificity of the HPr determination was checked

for various substances which might form a chromophore with p-dimethylaminobenzaldehyde. Proline, and various medium components such as Earle's balanced salt solution, phenol red, and Basal Medium Eagle (Diploid) without serum yielded negative results. The component of the medium which was found to contain much HPr was the fetal calf serum. Since the medium which had not been exposed to the WI 38 cells contained a great amount of HPr (approximately 30 times as much as the cells), it was questioned as to whether the cellular content was due to medium not rinsed from the cells when they were taken for a sample. The rinse from the cells was assayed for HPr and a negligible amount was found. Thus it was concluded that the HPr assay accurately determined the HPr content of the cells and was quite specific for this purpose.

#### Methods of Analysis of the Data

The data for each parameter studied occurs in three groups: (1) the fibroblasts grown to confluency representing generations 16 through 70; (2) the fibroblasts in the final degeneration phase which have ceased dividing; and (3) the SV<sub>40</sub> transformed WI 38 cells. The data from the fibroblasts grown to confluency was arbitrarily divided into 4 groups of 13 generations each so that the various ages of cells may be compared with degenerated and transformed cells. The mean, standard deviation, and standard error of the mean (Richmond, 1964) were determined for each group of data and values which

were two standard deviations from the mean were considered aberrant and were discarded (Richmond, 1964). Using the 16-29 group as a base, the Student t-test (Richmond, 1964) was used to determine significant differences between the groups.



## RESULTS

## RESULTS

### Growth of the Cell Strain

In the cultivation of the WI 38 fibroblasts, certain characteristics were observed. When a culture was trypsinized during subcultivation, the spindle-shaped cells pulled away from the glass and became amorphous masses. In 3 to 12 hours after subculturing, the fibroblasts settled onto the glass surface, adhered, and regained their spindle-shape. Within a few hours the culture was in the log phase of growth, and many cells were observed in mitosis. This was easily observed because the cells in mitosis became spherical as illustrated in Figure 3A. With cultures of the WI 38 fibroblasts in the early generations, i.e., before generation 45, mitosis continued until the fourth day after subculturing, at which time very few cells were observed in mitosis. The fibroblasts formed a confluent sheet as illustrated in Figure 3B and were presumably contact-inhibited from further cell division. This growth to confluency was a function of age of the culture, and in older cultures it occurred in 5 to 10 days.

The WI 38 cells have been demonstrated to be cultivated for  $50 \pm 10$  generations before final degeneration of the culture (Hayflick and Moorhead, 1961). The WI 38 fibroblasts used in this study were cultivated until generation 70. However, this was not considered different from the normal  $50 \pm 10$  generations (personal communication with Dr. L. Hayflick).

Figure 3A. Photomicrograph (250X) of the WI 38 fibroblasts in the log phase of growth illustrating cells in mitosis which are spherical (generation 32).

B. Photomicrograph (125X) of WI 38 fibroblasts in a confluent sheet (generation 32).



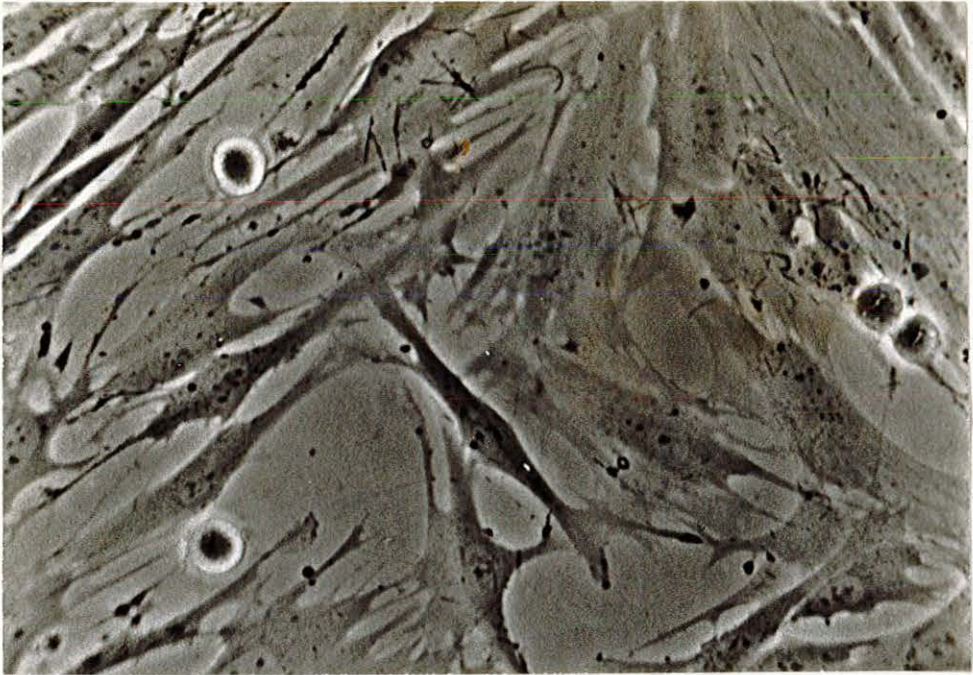


Figure 3A



Figure 3B



Upon microscopic examination, the fibroblasts were observed as elongated, spindle-shaped cells containing one nucleus with 1 to 4 dense nucleoli as illustrated in Figure 4A. Observation of the cytoplasm reveals many long filamentous structures believed to be mitochondria, and many small dense granules. A sheet of cells is composed of numerous swirls of fibroblasts, each swirl containing elongated cells oriented in a common direction, the cell orientation resulting from parallel alignment and varying from area to area.

In the late generation cells of Phase III illustrated in Figure 4B, degenerative changes slowly occurred. Much cellular debris accumulated in the medium and fine debris aggregated about the cell surfaces which presumably consists of protoplasmic material resulting from cell degeneration. Progressively more and more clear vacuoles appeared in the cytoplasm of the fibroblasts, presumably lysosomes, which correlates with the morphological studies of Robbins et al. (1970). When the cells finally stopped dividing during the degenerative phase, the fibroblasts were much less polarized, i.e., they were more spread out with a diffuse perimeter. Although mitotic activity ceased, the pH of the culture decreased indicating metabolic activity.

The SV<sub>40</sub> transformed WI 38 cells presented in Figure 5 are of the polygonal or epithelial morphology (Paul, 1970). The transformed cells are approximately one-half the size of the WI 38 fibroblasts, although occasionally large cells were

Figure 4A. Photomicrograph (1250X) of WI 38 fibroblasts showing internal structure.

B. Photomicrograph (250X) of WI 38 fibroblasts in Phase III (generation 69).



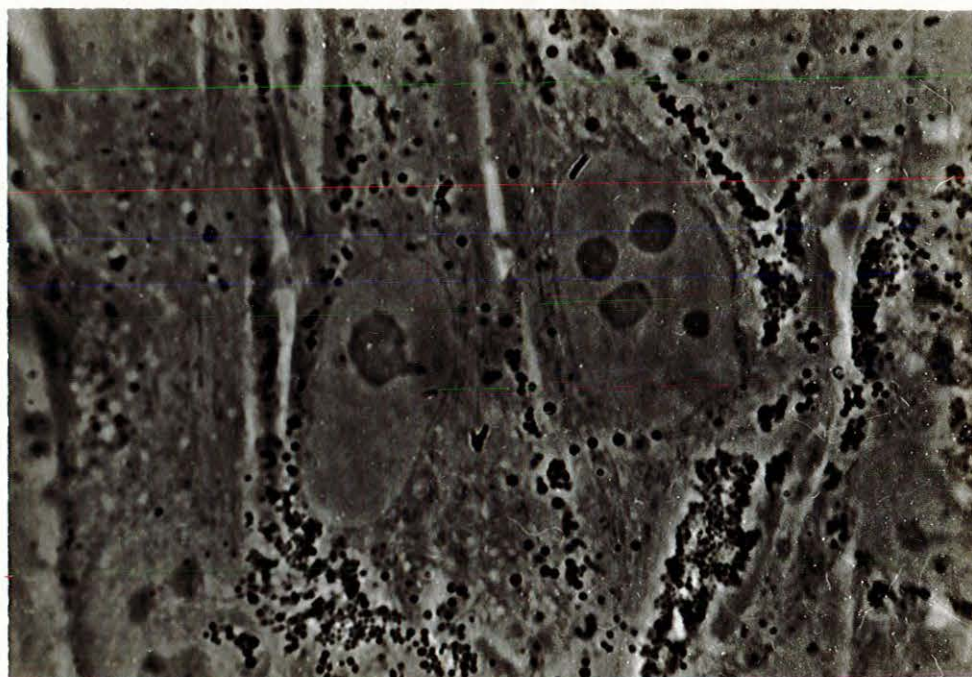


Figure 4A

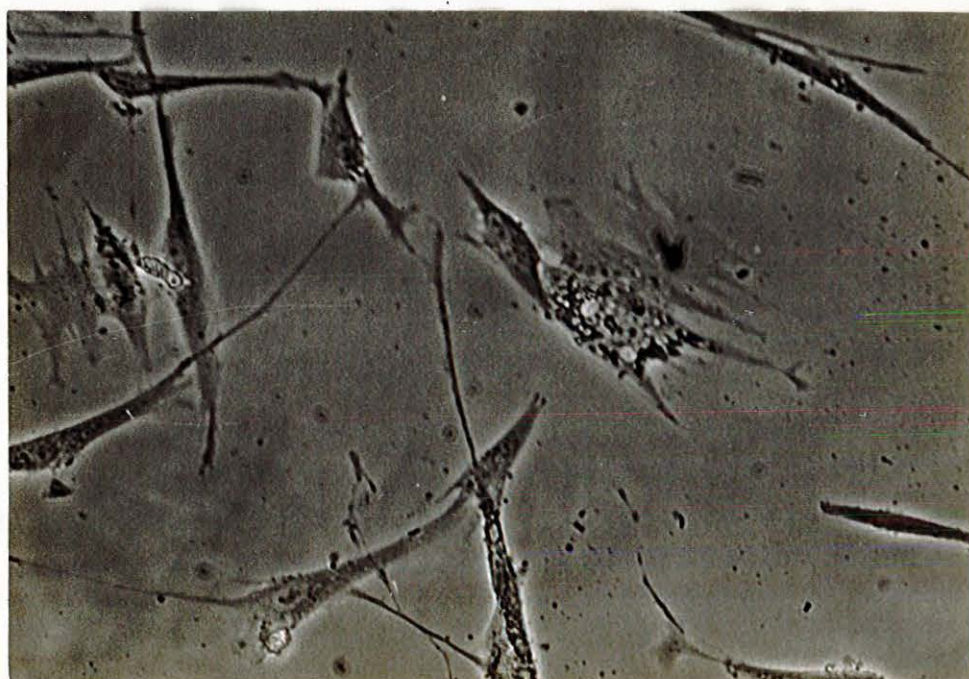


Figure 4B

Figure 5. Photomicrograph (250X) of SV<sub>40</sub>  
transformed WI 38 cells.

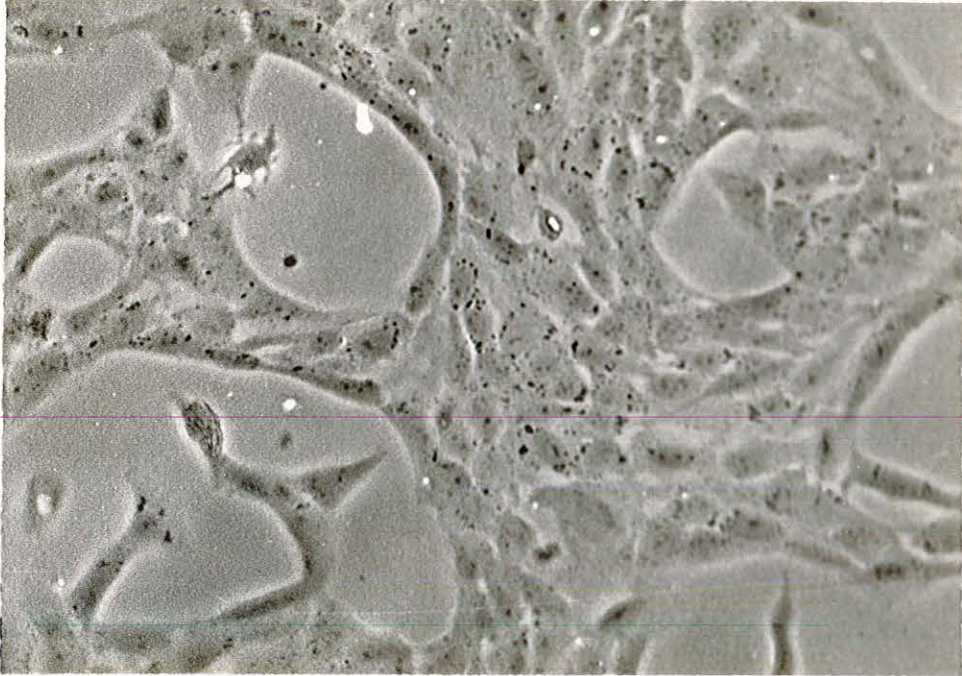


Figure 5



observed. The transformed cells were usually mononucleate, although occasionally multinucleate cells were observed. The nucleus contained 1 to 2 globular nucleoli and karyotypic analysis revealed them to be of heteroploid chromosomal configuration. The transformed cells grow apparently indefinitely in culture, and when subcultured 1 : 2, became confluent in 2 days and exhibited no contact inhibition. (Aberchrombie et al., 1954).

Cell counts were made of confluent cultures at various generations. Figure 6 indicates that the total number of cells in a confluent culture decreases as a function of increasing generations. A significant decrease ( $p < 0.01$ ) occurs after the generation group 50-60, i.e., during Phase III of the culture.

#### Cellular DNA Content

The results of the DNA content per  $10^6$  cells and per mg protein are presented in Table I and illustrated in Figure 7. No significant change was observed for cellular DNA content as a function of age in either confluent cultures or the degenerated cells. The mean value of the total DNA content per  $10^6$  cells for all generations of confluent cultures together with the degenerated cells is  $14.0 \pm 0.5$  ( $\pm$  standard error). The transformed cells showed a significant increase ( $p < 0.001$ ) over the normal fibroblasts, containing almost 3 times as much DNA.

Figure 6. The number of WI 38 fibroblasts  
in confluent cultures at various generations.

FIGURE 6

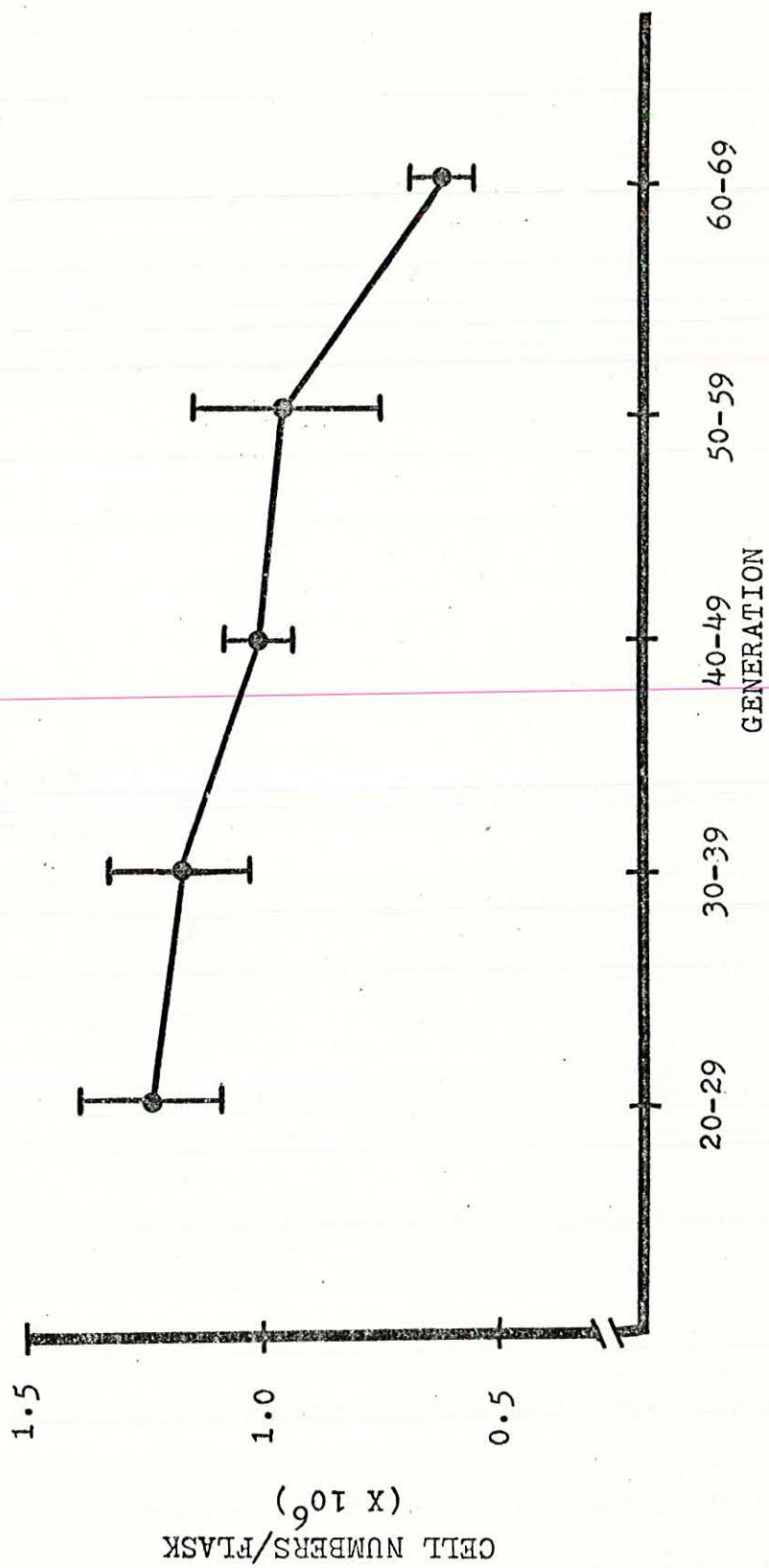




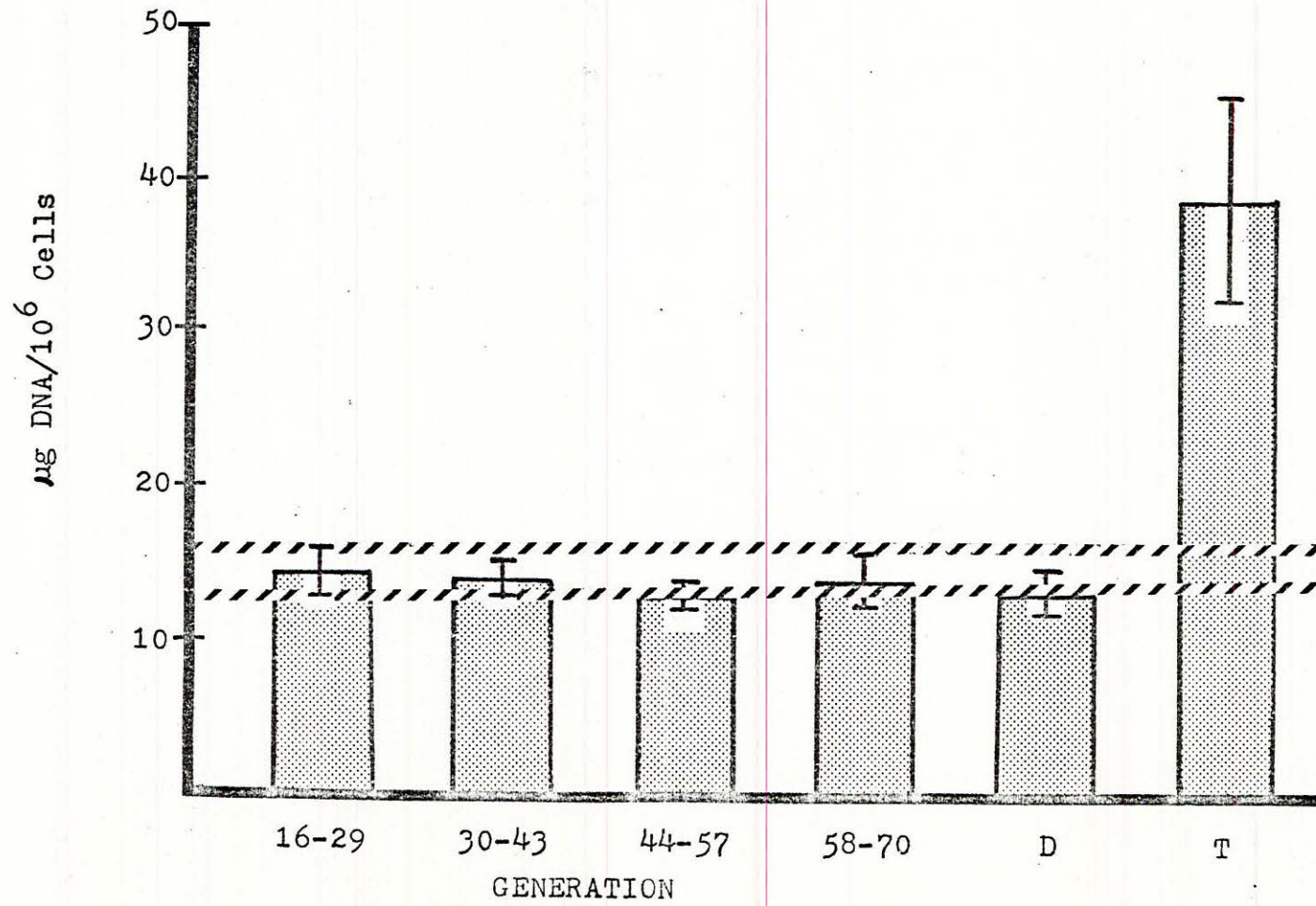
Table I. The Effects of Aging and Transformation on the DNA and RNA Content of WI 38 Fibroblasts

AGE (Generation)	DNA Content		RNA Content		* $\frac{\text{RNA}}{\text{DNA}}$
	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$	$\mu\text{g}$	
	$10^6$ Cells	mg Protein	$10^6$ Cells	mg Protein	
16-29	** $14.7 \pm 1.5$ n = 11	$93.3 \pm 18.6$ n = 11	$15.0 \pm 1.4$ n = 12	$79.1 \pm 14.1$ n = 10	$1.35 \pm 0.45$ n = 10
30-43	$14.6 \pm 0.9$ n = 11	$95.2 \pm 16.9$ n = 11	$15.5 \pm 1.0$ n = 11	$95.2 \pm 16.9$ n = 11	$1.08 \pm 0.17$ n = 11
44-57	$13.2 \pm 0.8$ n = 25	$87.2 \pm 12.2$ n = 10	$16.8 \pm 1.8$ n = 24	<sup>a</sup> $96.3 \pm 10.2$ n = 23	$1.36 \pm 0.13$ n = 23
58-70	$14.6 \pm 1.5$ n = 12	$88.1 \pm 18.6$ n = 10	$17.0 \pm 1.5$ n = 9	<sup>a</sup> $110.0 \pm 22.8$ n = 10	$1.32 \pm 0.17$ n = 10
D	$13.8 \pm 1.2$ n = 13	$66.2 \pm 8.7$ n = 12	<sup>a</sup> $22.2 \pm 2.4$ n = 12	<sup>a</sup> $102.4 \pm 3.0$ n = 11	$1.66 \pm 0.16$ n = 12
T	<sup>a</sup> $39.2 \pm 1.2$ n = 13	<sup>a</sup> $220.0 \pm 54.2$ n = 8	<sup>a</sup> $31.8 \pm 3.9$ n = 8	<sup>a</sup> $175.8 \pm 14.3$ n = 8	$1.02 \pm 0.65$ n = 8

n = number of experiments; D = degenerated cells; T = transformed cells  
 $\mu\text{g}/10^6$  Cells; \*\* $\pm$  standard error; <sup>a</sup><sub>p</sub> < 0.001

Figure 7. The effect of aging and transformation on the DNA content of fibroblasts.

FIGURE 7



D = degenerated cells; T = transformed cells



### Cellular RNA Content

The results of the RNA content per  $10^6$  cells presented in Table I and Figure 8 indicate a slight but insignificant increase in RNA content of confluent cultures as a function of age. In the degenerated cells a significant increase in RNA content ( $p < 0.001$ ) over the confluent cultures was observed.

The RNA/protein ratio demonstrated a significant increase in the 44-57 generation group ( $p < 0.001$ ), the 58-70 generation group ( $p < 0.001$ ), and in the degenerated cells ( $p < 0.001$ ), compared to the 16-29 generation group. However, the RNA/DNA ratios did not reflect this increase in RNA content in the aging cells.

In the transformed WI 38 cells, the RNA content was significantly higher ( $p < 0.001$ ), approximately two times that of the 16-29 generation group. However, the RNA/DNA ratio did not indicate an increase in the transformed cells, due to the increase in both RNA and DNA.

### Cellular Protein Content

From Table II and Figure 9 it can be seen that the protein content per  $10^6$  cells remained rather constant as a function of culture age. Also, no significant change was observed in the cellular content of protein for the transformed cells.

### Cellular Hydroxyproline Content

The HPr content per  $10^6$  cells as presented in Table II

Figure 8. The effect of aging and transformation on the RNA content of fibroblasts.

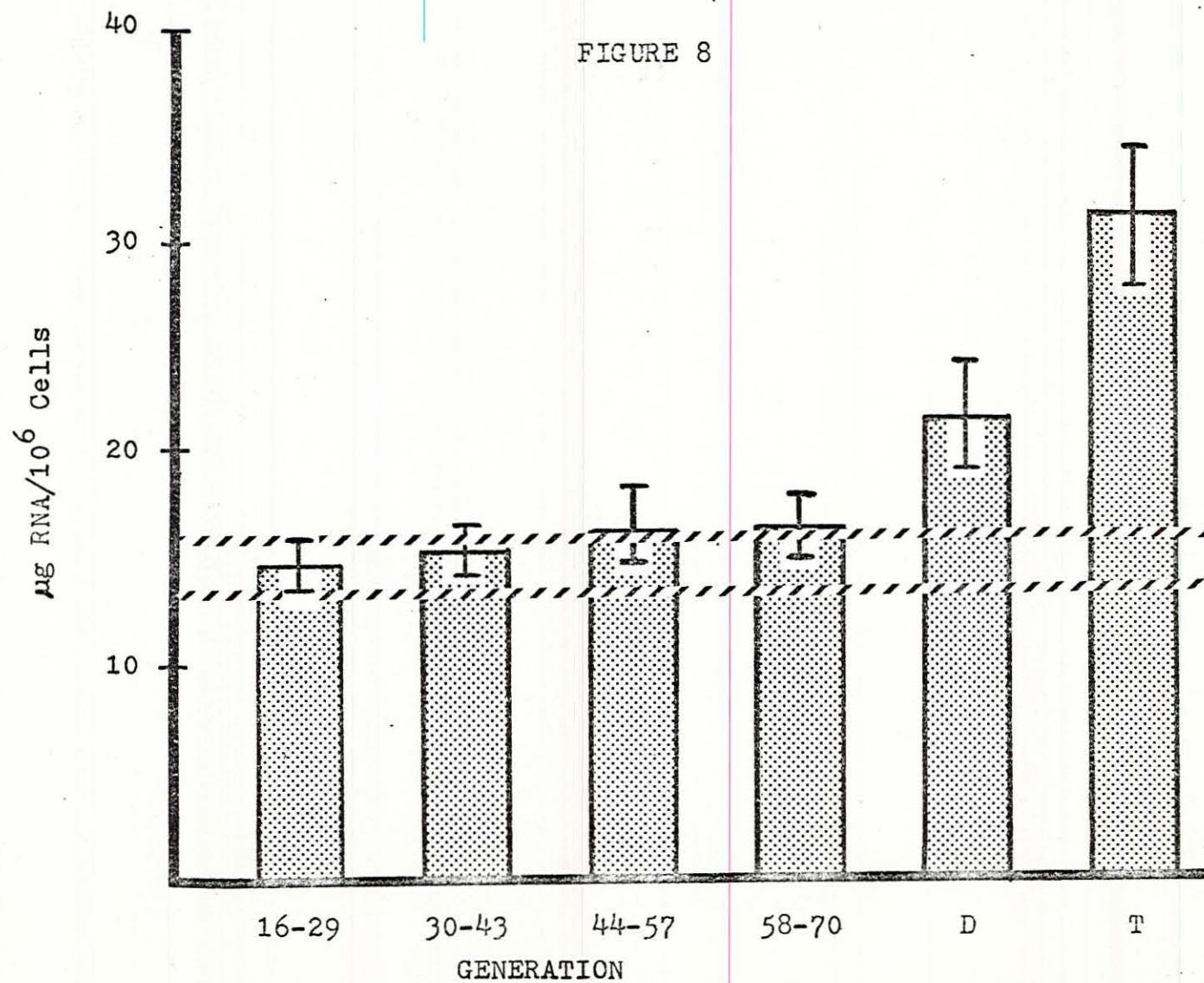


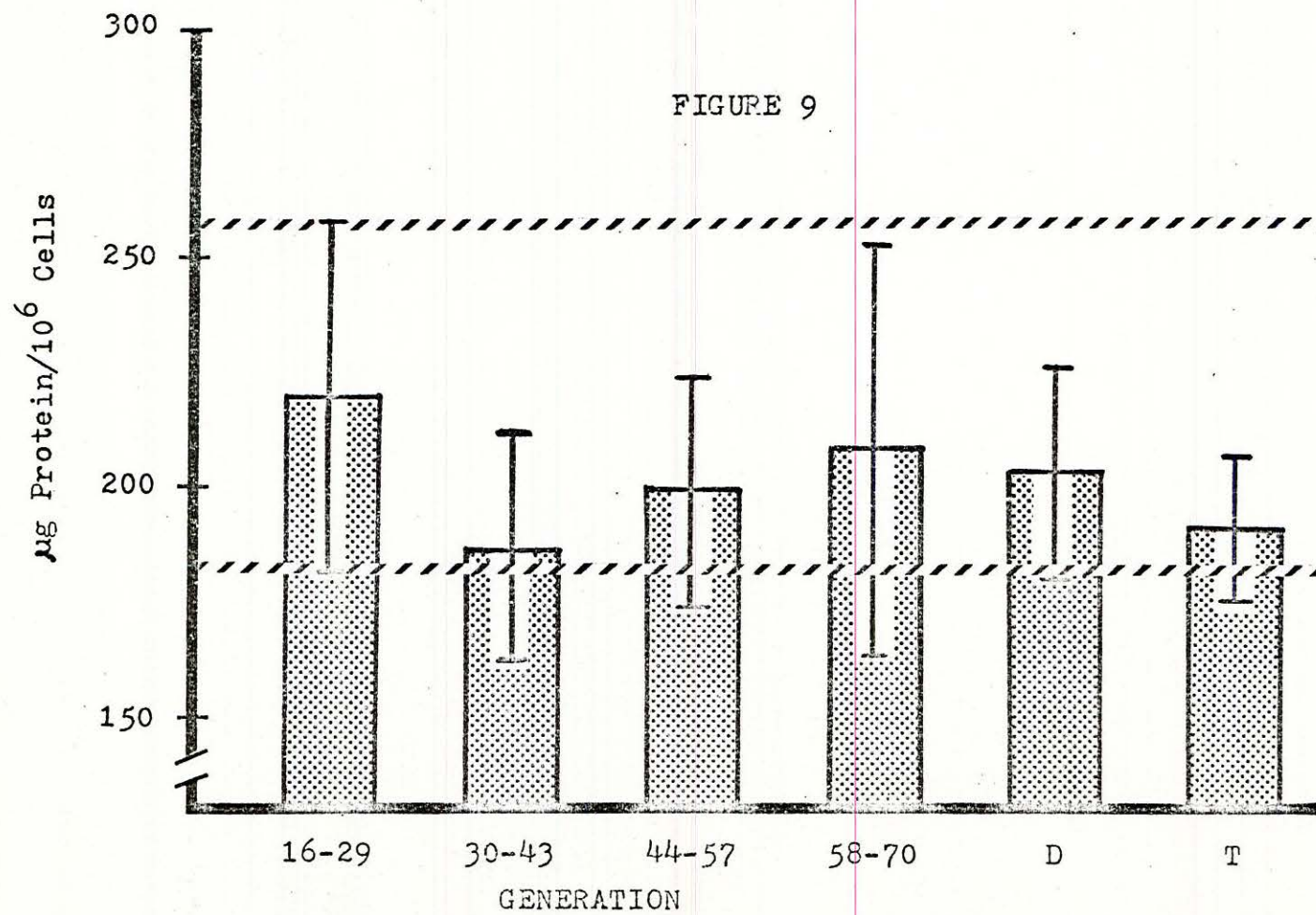


Table II. The Effects of Aging and Transformation on the Protein and Hydroxyproline Content of Fibroblasts

AGE (Generation)	Protein Content	HPr Content	
	$\mu\text{g}$	nmoles	nmoles
	$10^6$ Cells	$10^6$ Cells	mg Protein
16-29	*220.2 $\pm$ 38.5 n = 12	6.9 $\pm$ 0.6 n = 9	41.1 $\pm$ 6.8 n = 7
30-43	186.2 $\pm$ 26.2 n = 11	<sup>a</sup> 10.3 $\pm$ 0.7 n = 0.7	67.1 $\pm$ 19.8 n = 10
44-57	202.1 $\pm$ 24.9 n = 25	<sup>a</sup> 12.3 $\pm$ 1.1 n = 24	62.8 $\pm$ 14.3 n = 20
58-70	208.8 $\pm$ 46.9 n = 8	<sup>a</sup> 12.6 $\pm$ 3.4 n = 8	58.2 $\pm$ 12.7 n = 9
D	204.1 $\pm$ 22.8 n = 8	<sup>a</sup> 17.5 $\pm$ 2.8 n = 10	<sup>b</sup> 67.8 $\pm$ 4.1 n = 10
T	192.8 $\pm$ 14.6 n = 8	<sup>a</sup> 2.1 $\pm$ 1.0 n = 8	<sup>b</sup> 19.6 $\pm$ 7.2 n = 8

n = number of experiments; D = degenerated cells;  
T = transformed cells; \* $\pm$  standard error; <sup>a</sup> $p < 0.001$ ; <sup>b</sup> $p < 0.01$

Figure 9. The effect of aging and transformation on the protein content of fibroblasts.



D = degenerated cells; T = transformed cells



and Figure 10 increases as a function of age. When compared with the 16-29 generation group, there was a significant increase in HPr content in the 30-43 generation group ( $p < 0.001$ ), the 44-57 generation group ( $p < 0.001$ ), the 58-70 generation group ( $p < 0.001$ ), and in the degenerated cells ( $p < 0.001$ ). The HPr/protein ratios did not reflect this increase except in the degenerated cells ( $p = 0.01$ ).

It was shown that the transformed cells contained significantly less ( $p < 0.01$ ) HPr per  $10^6$  cells than the non-transformed fibroblasts. The HPr/protein ratio was also significantly decreased ( $p < 0.01$ ) compared to the normal fibroblasts.

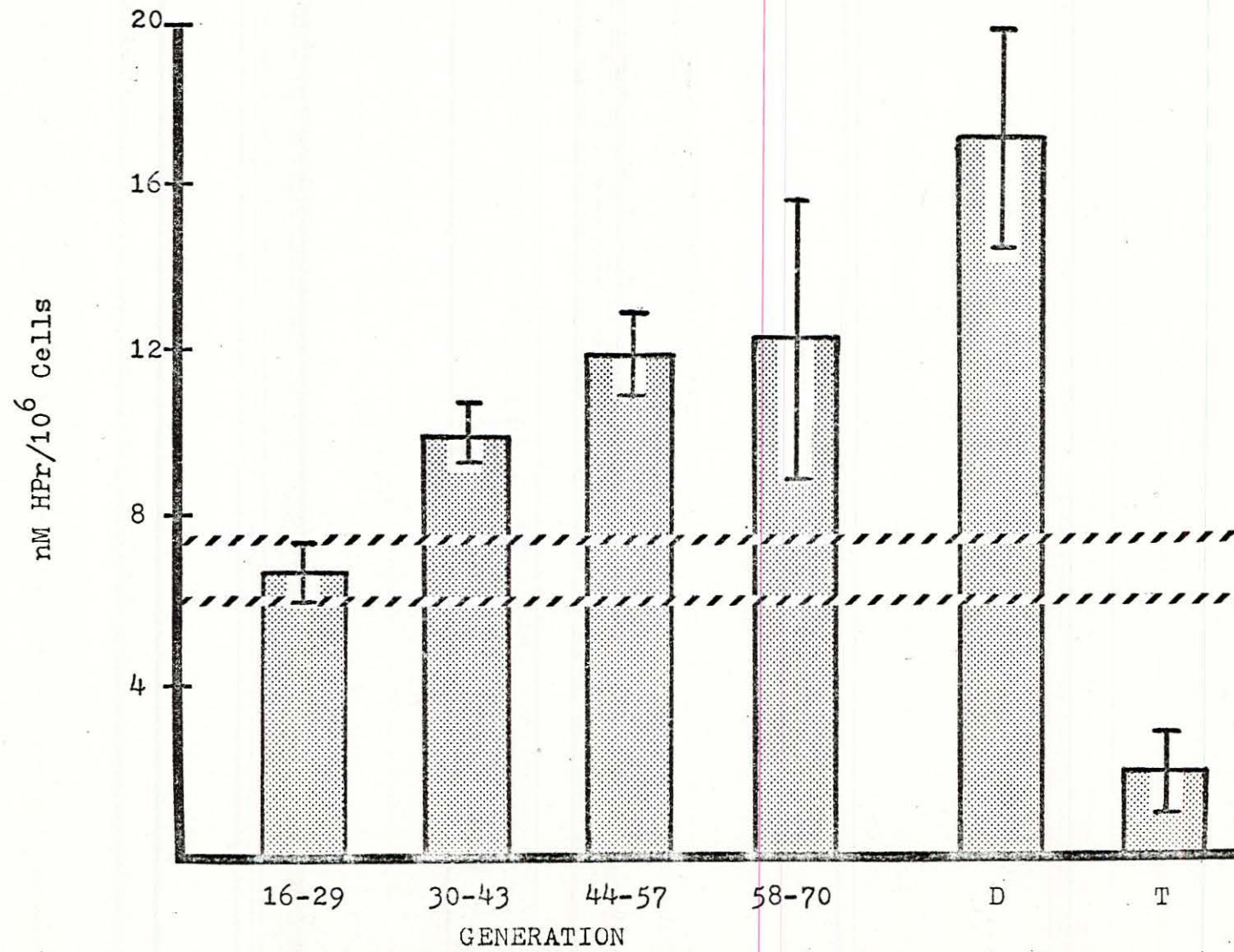
#### Hydroxyproline Content of the Medium

The medium which bathed the cells was subjected to the HPr determination. The control medium, i.e., medium which had not bathed cells, contained 384 nmoles HPr/12 ml. Since the HPr assay demonstrated approximately 20% variation with the cells, small changes in the medium HPr could not be detected by this method of analysis.

Medium which was not hydrolyzed with acid, i.e., the first step in the HPr determination, was assayed for free HPr. The control contained 210 nmoles HPr/12 ml of medium. With the high amount of free HPr and the variability of the assay, it was concluded that this method was not adequate for analysis.

Figure 10. The effects of aging and transformation on the HPr content of fibroblasts.

FIGURE 10



D = degenerated cells; T = transformed cells



## DISCUSSION

## DISCUSSION

In this study, the aging process was investigated at the cellular level using fibroblasts derived from human embryonic lungs as a model system. Cell counts were made of confluent cultures at various generations. It was observed that cell numbers significantly decreased in the Phase III cultures. Since the cultures with decreased numbers were observed to be confluent and they were confined to the area of the T-60 flask, it was presumed that the cells became larger with increasing age. It is possible that this is due to the increase in lysosomes (Robbins et al., 1970), lipid materials (Kritchevsky et al., 1970), and glycogen (Cristofalo et al., 1970). This correlates well with the size analysis of embryonic heart cells in culture (Simmons, 1970). It was demonstrated that as the heart cells underwent senescence in culture, their volume increased significantly. The cell volumes of the WI 38 fibroblasts have been measured (Cristofalo, 1970) and shown to increase significantly with age.

Routinely in the laboratory, two ages of WI 38 fibroblasts are cultivated at the same time, usually one of early generation and one old. On occasion, both are subcultured at the same time. It was observed that trypsinization frees the aged cells from the glass more rapidly than the younger cells. It was apparent that this observation may be due to a decrease in the connective tissue with which the cells adhere to the glass. However, this may be due to

a qualitative change in the connective tissue or simply to a decrease in cell numbers in the confluent aged cultures as compared to young confluent cultures

This previous observation, along with the work of Cristofalo et al. (1970) that RNA increased with age while the DNA and protein content of the fibroblasts remained constant, and the fact that collagen is the major protein produced by the fibroblasts, was the basis for the present study. It was questioned whether the increase of RNA in aged cells while maintaining the total protein constant, had an effect on the collagen content.

The DNA content of the WI 38 fibroblasts as measured in this study remained constant throughout the in vitro lifespan of the cells. This agreed with the work of Cristofalo et al. (1970). The mean value of the DNA content per  $10^6$  cells for all generations was  $14.0 \pm 0.5 \mu\text{g}$ . This is somewhat greater than that determined by Cristofalo et al. (1970),  $7.94 \pm 0.55 \mu\text{g}$  per  $10^6$  cells using the same method; and Rudkin et al. (1964),  $7.3 - 10.9 \mu\text{g}$  per  $10^6$  cells, using cytospectrophotometric methods. The value determined in this study is comparable to that of Tedesco et al. (1966) of  $15.4 - 15.9$  per  $10^6$  cells, using the same method with peripheral blood leucocytes and diploid human cells in culture.

This study demonstrated a significant increase in RNA content of the fibroblasts with age. These results are



comparable to, and parallel those of Cristofalo et al. (1970).

The hydroxyproline content of the fibroblasts is indicative of the collagen content because very little HPr is found anywhere else in the cell, and 10 out of every 100 amino acid residues in collagen is HPr (Gould, 1968). The results of the determination of the HPr content of the WI 38 fibroblasts shows an increase in HPr as the cells age. In previous work with human embryonic skin, gingiva, and umbilical fibroblasts (Macek et al., 1967), a constant HPr content was noted until generation 40 and then a gradual decline. In this same study, two other populations of embryonic skin fibroblasts showed no change in HPr content as the cells age. Houch et al. (1971) reported a great loss in the ability of the fibroblasts to synthesize collagen after generation 35. It is apparent that the results of the present study differ with previous work. Perhaps this is due to the difference in cell types or methods used.

The findings of this study, i.e., the increase in RNA content with a constant amount of DNA and protein as the cells age are in agreement with the work of Cristofalo et al. (1970). In the degenerated cells, the constant amount of DNA suggests that DNA synthesis is inhibited and this could account for the lack of cell proliferation. This also suggests that the synthesis of macromolecules proceeds independently of each other. To explain this, it may be hypothesized that as the cells age, there is an accumulation of

damage sites involved in the synthesis of RNA (Wulff et al., 1962), or part of the DNA is cross-linked (Bakerman, 1969), or mutations (Smith, 1962) take place, all of which produce a defective messenger RNA. This, in turn, produces enzymes incapable of normal function. This consequent accumulation of substrate molecules then results in derepression of RNA synthesis (Wulff et al., 1962), and more RNA is formed. This hypothesis could account for the heterogeneity of the cell cycle (Macieira-Coelho et al., 1966), the decreased cell proliferation in Phase III, and the final inhibition of mitosis in the degenerated cells. If cross-linkage of the DNA preventing "uncoiling" and replication were not the cause, then it is possible that the appropriate enzymes or proteins for cell division are not being produced.

The results of this study demonstrate that the HPr content of the WI 38 fibroblasts increases with cellular age. This indicates that the collagen content increases with age. Since the cellular protein content remained constant with age, this increase in collagen, which is a protein, was not detected in the protein determination. The method of protein determination was that of Lowry et al. (1951) which depends on the reaction of the tyrosine and tryptophan residues with the Folin-Ciocalteu reagent forming the chromophore. Since collagen has no tryptophan residues (Gould, 1968), and very few tyrosine residues, this was not detected by the Lowry procedure. It might be hypothesized that the increased



amount of RNA might cause the increase in HPr, as well as glycogen, lipid, and lysosomes, as the cells age.

The results of the DNA, RNA, protein, and HPr content of the transformed cells are as predicted in the Introduction. The DNA content was almost 3 times that of normal cells. This is due to the heteroploid chromosomal configuration. The RNA content is approximately twice that of the normal cells, probably for producing protein for cell division. The protein content is approximately the same in the transformed cells as the normal fibroblasts. This is perhaps due to the fact that the transformed cells are more active metabolically than the normal cells (Harris, 1964) and are more active in cell division. The HPr content is much less than the normal cells because of the absence of specialized function in transformed cells (Harris, 1964).

The scope of this investigation demonstrates quantitative changes in the parameters studied. However, changes might have taken place which could not be detected by the approach taken. For example, the protein content of the WI 38 fibroblasts was demonstrated to remain constant with age. By using radioactive tracers, the turnover of protein might be increased in aged cells where the RNA content has increased. The constant amount of protein may be due to the production of more protein and more enzymes which degrade it. The increase of HPr in older generation cells may be due to a decreased degradation of collagen.



The enzymes forming collagen may function normally but the enzymes which degrade it may have decreased activity.

Turnover rates may be determined by inhibiting enzymes. For example, an enzyme vital to the formation of collagen may be specifically inhibited, and measuring the collagen content before inhibition and at a time interval after would yield the rate of breakdown. Also, by blocking an enzyme vital to collagen degradation and measuring collagen content before and after inhibition would provide the rate of synthesis. From these two parameters, the turnover of collagen can be determined.

---

## SUMMARY

## SUMMARY

1. The aging process has been investigated using fibroblasts (WI 38) derived from human embryonic lungs as a model system for the study of cellular senescence.
2. The cellular DNA, RNA, protein, and hydroxyproline contents were measured in confluent cultures of various ages, degenerated cells, and cultures of SV<sub>40</sub> transformed WI 38 cells.
3. The cellular DNA content remained at a constant level in confluent cultures and degenerated cultures. The cellular RNA content increased somewhat in confluent cultures and increased significantly in degenerated cultures. The cellular protein content did not significantly vary in confluent cultures with age or in degenerated cells. The cellular HPr content increased in the confluent cultures with age and the most dramatic increase occurred in the degenerated cells.
4. In the SV<sub>40</sub> transformed WI 38 cells, the DNA content was almost 3 times as much as the non-transformed fibroblasts; the RNA content was almost 2 times that of normal cells; the protein content was approximately the same as the normal cells; and the HPr content was much less than the normal cells.



## BIBLIOGRAPHY

## BIBLIOGRAPHY

- Aberchrombie, M. and Heaysman, J.E.M., Observations on the Social Behavior of Cells in Tissue Culture. Exp. Cell Res. 6: 293, 1954.
- Bakerman, S., Aging Life Processes. Charles C. Thomas, Springfield, Illinois, 1969.
- Burton, K., A study of the Conditions and Mechanism of the Diphenylamine Reaction for the Colorimetric Estimation of Deoxyribonucleic Acid. Biochem. J. 62: 315, 1956.
- Carrel, A., and Burrows, M.T., Cultivation of Adult Tissues and Organs Outside of the Body. J.A.M.A. 55: 1379, 1910.
- Carrel, A., and Ebeling, A.H., Antagonistic Growth Principles of Serum and Their Relation to Old Age. J. Exp. Med. 37: 419, 1923.
- Cristofalo, V.J., Metabolic Aspects of Aging in Diploid Human Cells, In: Aging in Cell and Tissue Culture. Holecova, E., and Cristofalo, V.J., eds. Plenum Press, New York, 1970.
- Cristofalo, V.J., Animal Cell Cultures as a Model System for the Study of Aging. Adv. in Gerontol. Res. 4: 45, 1972.
- Cristofalo, V.J., Howard, B.V., and Kritchevsky, D., The Biochemistry of Human Cells in Culture. In: Organic Biological and Medicinal Chemistry. Gallo, U., and Santamaria, L., eds. 2:95, North Holland Publications, Amsterdam, 1970.
- Dische, Z., In: The Nucleic Acids. Chargaff, E., and Davidson, J.N., eds. Academic Press, New York, 1955.
- Earle, W. R., Production of Malignancy In Vitro. IV. The Mouse Fibroblast Cultures and Changes Seen in the Living Cells. J. Natl. Cancer Inst. 4: 165, 1943.
- Earle, W. R., Evans, V.J., Sanford, K.K., Shannon, J.E., and Watts, H.K., Influence of Inoculum Size on Proliferation in Tissue Culture. J. Natl. Cancer Inst. 12: 133, 1951.
- Gey, G.O., and Gey, M.K. The Maintenance of Human Normal Cells and Tumor Cells in Continuous Culture. Am. J. Cancer Res. 27: 45, 1936.

- Girardi, A.J., Jensen, F.C., and Koprowski, H., SV<sub>40</sub>-induced Transformation of Human Diploid Cells: Crisis and Recovery. J. Cell. and Comp. Physiol. 65: 69, 1965.
- Gould, B.S., Collagen Biosynthesis. In: Treatise on Collagen. 2A: 139, Gould, B.S., ed. Academic Press, New York, 1968.
- Harris, M., Cell Culture and Somatic Variation. Holt, Rinehart and Winston, New York, 1964.
- Hay, R.J., and Strehler, B.L., Cell Strain Senescence In Vitro. Exp. Gerontol. 2: 123, 1967.
- Hayflick, L., The Limited In Vitro Lifetime of Human Diploid Cell Strains. Exp. Cell Res. 37: 614, 1965.
- Hayflick, L., and Moorhead, P.S., The Serial Cultivation of Human Diploid Cell Strains, Exp. Cell Res. 25: 585, 1961.
- Houck, J.C., Sharma, V.K., and Hayflick, L., Functional Failures of Cultured Human Diploid Fibroblasts After Continued Population Doublings. Proc. Soc. Exp. Biol. and Med. 137: 331, 1971.
- Kritchevsky, D., and Howard, B.V., Lipid Metabolism in Human Diploid Cells. In: Aging in Cell and Tissue Culture. Holeckova, E., and Cristofalo, V.J., eds. Plenum Press, New York, 1970.
- Krohn, P.L., Aging. Science. 149: 391, 1966.
- Krohn, P.L., Review Lectures on Senescence. II. Heterochronic Transplantation in the Study of Aging. Proc. Roy. Soc. B. 157: 128, 1962.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. 193: 265, 1951.
- Macek, M., Hurych, J., and Chvapil, M., The Collagen Protein Formation in Tissue Cultures of Human Diploid Strains. Cytologia. 32: 426, 1967.
- Macieira-Coelho, A., Ponten, J., and Philipson, L., The Division Cycle and RNA-Synthesis in Diploid Human Cells at Different Passage Levels In Vitro. Exp. Cell Res. 42: 673, 1966.
- Mejbaum, W., Uber die Bestimmung Kleiner Pentosemengen Insbesondere in Derivaten der Adenylsaure. Ztschrg. F. Physiol. Chem. 258: 117, 1939.



- Merchant, D.J., Kahn, R.H., and Murphy, W.H., Handbook of Cell and Organ Culture. Burgess Pub. Co., Minneapolis, 1964.
- Parker, R.C., Methods in Tissue Culture. Harper and Row, New York, 1961.
- Paul, J., Cell and Tissue Culture. E. and S. Livingstone. Ltd., Edininburgh, 1960.
- Prockop, D.J., and Udenfriend, S., A Specific Method for the Analysis of Hydroxyproline in Tissue and Urine. Anal. Biochem. 1: 228, 1960.
- Richmond, S.B., Statistical Analysis. The Ronald Press Co., New York, 1964.
- Robbins, E., Levine, E.M., and Eagle, H., Morphologic Changes Accompanying Senescence of Cultured Human Diploid Cells. J. Exp. Med. 131: 1211, 1970.
- Rudkin, G.T., Hungerford, D.A., and Nowell, P.C., DNA Contents of Chromosome Ph<sup>1</sup> and Chromosome 21 in Human Chronic Granulocytic Leukemia. Science. 144: 1229, 1964.
- Ryan, J.M., and Quinn, L.Y., Nucleohistone Content During Aging in Tissue Culture. In Vitro. 6: 269, 1971.
- Schmidt, G., and Thannhauser, S.J., A Method for the Determination of Desoxyribonucleic Acid, Ribonucleic Acid, and Phosphoproteins in Animal Tissues. J. Biol. Chem. 161: 83, 1945.
- Simons, J.W.I.M., A Theoretical and Experimental Approach to the Relationship between Cell Variability and Aging In Vitro. In: Aging in Cell and Tissue Culture. Holeckova, e., and Cristofalo, V.J., eds. Plenum Press, New York, 1970.
- Smith, M.J., Review Lectures on Senescence. I. The Causes of Aging. Proc. Roy. Soc. B. 157: 115, 1962.
- Tedesco, T.A., and Mellman, W.J., Desoxyribonucleic Acid Assay as a Measure of Cell Number in Preparations From Monolayer Cell Cultures and Blood Leucocytes. Exp. Cell Res. 45: 230, 1966.
- Walford, R.L., The Role of Autoimmune Phenomena in the Ageing Process. In: Symposia of the Society for Experimental Biology. XXI. Aspects of the Biology of Aging. Academic Press Inc., New York, 1967.

Wulff, V.J., Quastler, H., and Sherman, F.G., An Hypothesis  
Concerning RNA Metabolism and Aging. Proc. Natl. Acad.  
Sci. 48: 1373, 1962.